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# MYELOID CELL PROMOTER AND CONSTRUCTS CONTAINING SAME

## TECHNICAL FIELD

The present invention relates to a novel promoter isolated from a myeloid cell, as well as *cis*-acting elements from the myeloid cell, and to novel constructs containing the same.

## BACKGROUND OF THE INVENTION

The expression of foreign genes in various cell types has become a commonplace occurrence in the field of biotechnology. The expression of the foreign, or heterologous, gene is performed by the transcription of the genetic information in the gene from DNA to RNA. The RNA is then translated into the protein for which the gene encodes.

In transcription, the RNA is synthesized using the DNA of the gene for a template. The RNA is synthesized by a reaction catalyzed by RNA polymerases. The RNA polymerase binds to a particular molecular site on the DNA to initiate the transcription process. This site to which the RNA polymerase binds is known as the promoter.

Therefore, in order to express heterologous genes in foreign cells, the genes must be under the control of a promoter. Although numerous promoters for expressing foreign genes have been taught in the literature, these promoters have generally failed to function when used to express genes within myeloid cells. Therefore, a need exists for promoters which may be used to express genes within such cells.

Integrins are a large family of cell surface glycoproteins that are heterodimers comprised of $\alpha$ and $\beta$ chain subunits. The promoters of integrins are

of special interest as these promoters may be used to express genes in a myeloid-specific manner. This is especially useful, as typical promoters which are used in genetic research, such as retroviral promoters, become repressed after being introduced into myeloid cells.

Genes linked to myeloid specific promoters may be used in a wide variety of applications. These applications include use in the development of cancer vaccines, as well as being used for screening compounds for their effect on myeloid cell specific gene expression.

A need therefore remains for the identification and isolation of myeloid cell specific promoters.

## SUMMARY OF THE INVENTION

The present invention relates to myeloid cell specific gene expression. The invention includes myeloid cell specific gene expression under the control of a myeloid cell specific promoter, preferably the CD11d promoter.

A preferred myeloid cell specific promoter is all or a functional portion of isolated or recombinant SEQ ID NO:1, such that the sequence is sufficient to direct myeloid cell specific expression of a gene. More preferably, the myeloid cell specific promoter of the present invention is the -946 to +74 region that is upstream (5') of the CD11d gene, as identified by SEQ ID NO:1. In particular, the present invention relates to portions of this sequence that are sufficient to direct myeloid cell specific expression of a heterologous gene, and also includes modifications of this sequence that retain sufficient activity to direct myeloid cell specific expression of a heterologous gene. More preferably, the invention relates to the region from -173 to +74 that is upstream (5') of the CD11d gene. The invention further includes all or a functional portion of isolated or recombinant SEQ ID NO:1, wherein the sequence comprises one or more modifications, such that the modified sequence retains sufficient activity to direct myeloid cell specific expression of a gene.

The invention further relates to a *cis*-acting element that influences the activity of the myeloid cell specific promoter. This influence may be either to increase or decrease the activity of the myeloid cell specific promoter. The *cis*-acting element comprises a portion of SEQ ID NO:1 that is sufficient to influence the activity of the myeloid cell specific promoter. In particular, the present invention relates to portions of this sequence that are sufficient to function as a *cis*-acting element that influences the activity of the myeloid cell specific promoter, and also includes modifications of this element that retain sufficient activity to influence the activity of the myeloid cell specific promoter.

A preferred *cis*-acting element is the −173 to +74 region that is upstream (5') of the CD11d gene as shown in SEQ ID NO:1. Also preferred is the −72 to −40 region, more specifically, the −63 to −40 region, that is upstream (5') of the CD11d gene. In particular, the invention is directed to *cis*-acting elements that positively influence the CD11d promoter, increasing the activity of the promoter.

*Cis*-acting elements that negatively influence the CD11d promoter are also incorporated as part of the present invention. Particularly, the present invention relates to the −591 to −378 region that is upstream (5') of the CD11d gene as shown in SEQ ID NO:1. This *cis*-acting element contains a cell-specific silencer element.

The invention also relates to the approximately 12 kb sequence that is upstream (5') of the CD11d gene as shown in SEQ ID NO:3. This sequence further comprises additional *cis*-acting elements which influence the activity of the myeloid cell promoter of the present invention.

Additionally, the invention includes constructs that contain DNA sequences sufficient to direct myeloid cell specific expression of a gene. These constructs comprise both the promoter of the present invention and a heterologous gene, whereby the expression of the heterologous gene is under the control of the myeloid cell specific promoter. The invention is also directed to constructs that contain one or more *cis*-acting elements of the present invention together with the promoter of the present invention, both linked to a heterologous gene, whereby the

expression of the heterologous gene is under the control of the myeloid cell specific promoter of the present invention, and the promoter is influenced by the *cis*-acting element(s) of the present invention.

The invention also includes cells that have been transfected with a construct of the present invention.

In addition, the present invention relates to methods of producing a selected heterologous gene product in a myeloid cell. These methods include introducing into the myeloid cell a heterologous gene under the control of a myeloid cell specific promoter, or a *cis*-acting element that influences the activity of the myeloid cell specific promoter, or both. The invention also includes cells produced by the methods described herein.

The present invention also relates to a method for identifying factors that can regulate myeloid cell specific transcription.

Further, the invention relates to a method of expressing a selected heterologous gene product in myeloid cells of an individual, i.e., gene therapy. According to this embodiment, cells produced by the methods described herein are introduced into an individual, wherein they express a heterologous gene under transcriptional control of a myeloid cell specific promoter or a *cis*-acting element that influences the activity of the cell specific myeloid promoter, or both.

The present invention provides a means of insuring that a selected product, such as a diagnostic, therapeutic or prophylactic substance, is expressed from a specific cell type, *in vivo*. Therefore, the present invention is useful, for example, for gene therapy or to drive the expression of antiviral agents, such as anti-HIV constructs. The present invention also provides novel promoters which may used to produce cancer vaccines. The present method is also useful in research, for example, to test the effect of the specific expression of heterologous genes, such as oncogenes, in specific cell types. Cells of the present invention are also useful for screening compounds for their effect on myeloid cell specific gene expression.

Further objects, features and advantages of the invention will be apparent from the following detailed description when taken in conjunction with the accompanying drawings.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the DNA sequence of the CD11d 5'flanking region and exons 1-7 and shows the sequence analysis data for the -946 to +74 CD11d promoter region (SEQ ID No: 1). The complete sequence in lowercase for introns 1, 3 and 4, and the sequence at the intron-exon junctions for introns 2,5, 6 and 7 and their sizes are shown. Consensus gt/ag splice junctions are underlined. Putative binding sites for transcription factors are underlined and indicated above the sequence. The transcriptional start site (+1), the ATG translational start site (boxed), and protein sequence are shown.

FIG. 2 depicts a CD11d restriction map and location of exons. The location of the translational stop codon (TGA) for CD11d is shown. The direction of transcription of CD11c and CD11d is indicated with arrows. CD11d exons 1-7 are depicted as thickened vertical lines below the location of restriction enzyme sites for *Eco*RI (E), *Hin*diii (H), X*bal* (X), SacI (S), and *Bam*HI (B). The reference point for the scale in bp is the CD11d transcriptional start site assigned as +1.

FIG. 3 shows the determination of the transcriptional start site for the CD11d gene with a schematic summarizing the sequencing gel information for the results of the transcriptional assays.

FIG. 4 is a graph illustrating that PMA downregulates the CD11d promoter. CD11d promoter activity in PMA-stimulated cells is expressed relative to that in unstimulated cells after correction for differences in transfection efficiencies.

FIG. 5 is a graph illustrating that the effect of PMA on the CD11d promoter is cell specific. The extent of CD11d 5'-flanking sequence in each construct shown is indicated. Promoter activity of each construct is expressed as

X-fold increase in activity above the background activity conferred by the promoterless control plasmid pGL3-Basic after correction for differences in transfection efficiencies. Expression of CD11c-*luc* is shown for comparison. The mean luciferase activities +/- the standard deviations are indicated.

FIG. 6 is a set of graphs illustrating that the Sp1-binding site is essential for CD11d promoter activity. The mean luciferase activities +/- the standard deviations are indicated.

FIG. 7 is a set of graphs showing the induction of the CD11d promoter with Sp1. The mean luciferase activities +/- the standard deviations are indicated.

FIG. 8 is a sequence schematic summarizing the results of gel analysis illustrating the loss of Sp1-binding *in vivo* in PMA-stimulated myeloid cells. The open and gray circles below the sequence refer to the guanidine residues on the non-coding strand. (SEQ ID NO:5 and 6 are shown.)

FIG. 9 shows the DNA sequence of the approximately 11.5 kb region located upstream (5') of the region coding for CD11d, CD11d exons and introns 1-7 for CD11d (SEQ ID No: 3).

## DETAILED DESCRIPTION OF THE INVENTION

The following defined terms are used throughout the application:

***Cis*-acting element**: A term used to describe a component of a DNA sequence which influences the activity of the same or immediately adjacent DNA sequence. For example, the DNA sequences to which transcription factors bind within promoters are said to be *cis*-acting control elements as they effect the expression of the adjacent gene or gene on the same chromosome.

**CD11**: A family of four leukocyte-associated single chain molecules that consist of two polypeptide chains; the larger of these chains ($\alpha$) is different for each member of the family and includes CD11a, CD11b, CD11c and CD11d; the smaller chain ($\beta$) is common to all four large chain molecules.

**Construct**: Recombinant DNA sequences, both linear and circular, that comprise a functional portion of the myeloid cell specific promoter and/or *cis*-acting element(s) together with DNA encoding a heterologous gene. The myeloid cell specific promoter and/or *cis*-acting element(s) are functionally linked to the heterologous gene in the constructs described herein.

**Functional portion**: refers to DNA sequences which are of sufficient size and sequence to have a desired function. In the present application, the desired function is the ability to cause tissue specific expression of a heterologous gene, or the ability to influence the activity of a promoter of the present invention.

**Heterologous gene**: Genes or DNA sequences which are not normally present in the cell as obtained, or which are not ordinarily functionally associated with a myeloid cell specific promoter region in the cell as obtained, or which are not ordinarily functionally associated with a myeloid cell specific *cis*-acting element that influences the activity of the cell specific myeloid promoter in the cell as obtained.

**Hybridizes**: A DNA hybridizes to a second DNA if the first DNA has sufficient nucleic acid sequence complementarily to the second DNA to allow the formation of base pairing and hydrogen bonding under standard DNA hybridization conditions between the two DNAs.

**Myeloid cell**: Includes monocytes, granulocytes, macrophages, mast cells, erythrocytes, dendritic cells and natural killer (NK) cells, and precursor cells in these cell lineages.

**Myeloid cell specific promoter**: A DNA sequence which functions as a transcriptional control element or elements and which directs the expression of a gene which is expressed in myeloid cells and which is expressed at reduced efficiency or not at all in other cell types and can include all or a portion of a DNA sequence which functions as a transcriptional control element.

**Substantially similar:** Two sequences that have a substantial degree of DNA or RNA sequence homology to each other.

The present invention is based on the isolation of a myeloid cell specific promoter and the demonstration that this promoter directs myeloid specific expression of a heterologous gene in transfection assays *in vivo,* and that the promoter is not capable of directing expression of heterologous genes in cancer cells. The particular myeloid cell specific promoter of the present invention is the CD11d promoter.

The CD11d promoter of the present invention is all or a functional portion of isolated or recombinant SEQ ID NO:1, such that the sequence is sufficient to direct myeloid cell specific expression of a gene.

It has been surprisingly and unexpectedly discovered that the–946 to +74 region of SEQ ID NO:1, as illustrated in FIG. 1., contains the promoter activity for the CD11d gene. More specifically, it has been surprisingly and unexpectedly discovered that the –173 to +74 region is sufficient to confer leukocyte-specific expression of a gene in myeloid cells, when the gene is a heterologous gene, such as a luciferase reporter gene.

These sequences which have CD11d promoter activity may further be modified. These modifications may be obtained by mutation, deletion and/or addition of one or more nucleotides in relation to the native sequence. They can be introduced in particular in order to improve the promoter activity, to suppress a transcription inhibiting region, to make a constitutive promoter regulatable or vice versa, to introduce a restriction site facilitating subsequent cloning steps, to eliminate the sequences which are not essential to the transcriptional activity, and the like. Such modifications may be made by any means well known in the art. See., Noti *et al.*, Molec. Cell. Biol. 16:2940-50 (1996) and Noti *et al.*, Molec. Immunol. 33:115-127 (1996), for methodologies using PCR to create and/or modify sequences.

The modified sequences may be screened for activity to determine if they retain the CD11d promoter activity. Such screening methodologies are well known in the art, and are further described in the following Examples.

The present invention is further based on the discovery of one or more *cis*-acting elements present within SEQ ID NO:1 that influence the CD11d promoter activity. Specifically, it has been discovered that several *cis*-acting elements are present upstream (5') of the CD11d gene that influence the CD11d promoter. This influence may be either to increase or decrease the activity of the myeloid cell specific promoter. The *cis*-acting element comprises a portion of SEQ ID NO:1 that is sufficient to influence the activity of the myeloid cell specific promoter. In particular, the present invention relates to portions of this sequence that are sufficient to function as a *cis*-acting element that influences the activity of the myeloid cell specific promoter, and also includes modifications of this sequence that retain sufficient activity to influence the activity of the myeloid cell specific promoter. The modifications would be created and screened in manners similar to those used to identify the promoter modifications as previously described.

Introns are known in the art to often have regulatory sequences contained therein. The invention contained herein also comprises the discovery of introns 1-6 which are shown in FIG. 9 (SEQ ID NO:3). The regulatory sequences contained within these introns can be determined by means well known in the art.

A preferred *cis*-acting element is the -173 to +74 region that is upstream (5') of the CD11d gene as shown in SEQ ID NO:1. This *cis*-acting element regulates cell-specific downregulation of CD11d by 4-phorbol-12-myristate 13-actetate (PMA). Also preferred is the -72 to -40, more specifically, the -63 to -40 region that is upstream (5') of the CD11d gene. This *cis*-acting element binds the transcription factor Sp1. It has been found that purified Sp1 binds at the -63 to -40 region that is upstream (5') of the CD11d gene; whereas crude protein extract that contains Sp1 binds to a larger region, namely, the -72 to -40 region that is upstream (5') of the CD11d gene. It has been discovered that a functional Sp1 binding site is necessary for high levels of CD11d promoter activity. The presence of these *cis*-acting elements positively influence the CD11d promoter, increasing the activity of the promoter.

It has also been discovered that Sp3 also influences upregulation of the CD11d promoter. Binding of Sp3 occurs in the regions identified in FIG. 9 (SEQ ID NO:3).

*Cis*-acting elements that negatively influence the CD11d gene are also incorporated as part of the present invention. Most notably, it has been discovered that the –591 to –378 region of SEQ ID NO:1 contains a cell-specific silencer element. As described in the following Examples, evidence of a cell-specific silencer element within the -591 to -378 region that serves to downregulate CD11d expression in Jurkat and IM-9 cells was found.

Additional cell-specific *cis*-acting elements that lie upstream of –944 of SEQ ID NO:1 are also part of the present invention. These *cis*-acting sequences lie within the approximately 12.5 kb region described in SEQ ID NO:3. Determination of these *cis*-acting elements is performed as described in the following Examples, or by any other means well known in the art.

The myeloid cell specific promoter(s) and *cis*-acting element(s) of the present invention can be obtained from a naturally-occurring source, or they can be produced using any of a variety of techniques, such as genetic engineering or cloning methods, PCR amplification or other synthetic techniques well known in the art.

The CD11d promoter is a strong promoter of expression in myeloid cells. Therefore, in addition to cell specific expression, the CD11d promoter offers the advantage of high level of expression of a desired product in myeloid cells.

Further the invention includes an isolated nucleic acid strand that hybridizes to either a nucleic acid strand having the sequence listed above (SEQ ID NO: 1) or its complement, and constructs containing such isolated nucleic acid strands.

Additionally, the invention includes constructs that contain DNA sequences sufficient to direct myeloid cell specific expression of a gene. These constructs include constructs comprising both the promoter of the present invention and a heterologous gene, whereby the expression of the heterologous gene is under

the control of the myeloid cell specific promoter. These constructs are constructed by methods well known in the art, including those exemplified in the following Examples.

Accordingly, the present invention also extends to a construct comprising a DNA sequence having CD11d promoter activity and/or *cis*-acting element activity according to the invention and a gene of interest placed under its control. It goes without saying that a construct according to the invention may contain several genes of interest either within the framework of a multicistronic construct (schematically represented by the "*cis*-acting element and/or promoter-gene 1-gene 2 . . . " arrangement) in which the different genes are placed downstream of the *cis*-acting element and/or the promoter according to the invention and are separated from each other by appropriate sequences, such as the IRES (for Internal Ribosome Entry Site) elements allowing the reinitiation of translation or alternatively within the framework of a bidirectional construct ("gene 1-promoter-gene 2") in which a *cis*-acting element and/or promoter according to the invention is inserted between two genes of interest in order to control their expression simultaneously.

For the purpose of the present invention, a gene of interest may be derived from a eukaryotic or prokaryotic organism or from a virus. It can be isolated by any conventional molecular biology technique or can be synthesized by the chemical route.

Moreover, a gene of interest may encode a polypeptide of interest corresponding to all or part of a protein as found in nature (native or truncated protein). It may also be a chimeric protein, for example coming from the fusion of polypeptides of diverse origins or from a mutant exhibiting improved and/or modified biological properties. Such a mutant can be obtained by conventional molecular biology techniques. Among the proteins or polypeptides of interest, there may be mentioned by way of nonlimiting examples: cytokines and growth factors.

A construct according to the invention may, in addition, comprise additional elements necessary for the expression of the gene of interest (intron

sequence, transcription terminator sequence and the like) or alternatively for its maintenance in the host cell considered. Such elements are known to persons skilled in the art.

The invention also includes cells that have been transfected with a construct of the present invention. The cells are transfected by the constructs of the present invention by any means well known in the art, including electroporation as taught in the following Examples. Other well known methods of transfecting cells with constructs of the present invention include liposome-mediated transfections and $CaPO_4$-mediated transfections.

A host cell to be transfected according to the present invention may be derived from cells which include, but are not limited to, myeloid cells – HL60, THP1 (both macrophage cells), dendritic cells, T cells (Jurkat, RH9 cells), and B cells (IM9, Burkitt Daudi cells).

The present invention further relates to a method of transfecting a myeloid cell line which comprises contacting a suspension of the cells with a heterologous gene construct, and exposing the cells to electroporation. In one embodiment of the invention the heterologous gene construct is a plasmid. In the preferred embodiment, the plasmid is a luciferase vector, such as firefly luciferase driven by the early SV40 enhancer/promoter.

The present invention is based, in part, on the discovery of an efficient technique for transfecting myeloid cell lines, and the discovery that the human genomic region upstream of (5' of) the genomic region encoding CD11d comprises a sequence which contains a control element(s) which is capable of directing myeloid specific transcription of a heterologous gene in transfected myeloid cells. This sequence is also capable of directing reduced, but good, expression of genes within T and B cells, and reduced expression of genes in epithelial cells.

The present invention further includes a method for identifying factors which can regulate myeloid cell specific transcription. This method comprises (a) obtaining a myeloid cell which contains (e.g., has been transfected

with, or is derived from a cell transfected with) a heterologous gene under the transcriptional control of a myeloid cell specific promoter (i.e., a myeloid cell promoter-heterologous gene construct); (b) contacting the cell containing the myeloid cell promoter-heterologous gene construct with a selected factor; and (c) assaying for expression of the heterologous gene and comparing its expression in cells contacted with the factor with expression of the gene in cells not contacted with the factor, and thereby determining whether the expression pattern of the heterologous gene is altered in cells contacted with the factor as compared to that of cells which have not been contacted with the factor. The construct and transfected cells are prepared as previously described. The assay is performed as described in the following examples.

Myeloid cell specific promoter-heterologous gene constructs, *cis*-acting element-heterologous gene constructs, or *cis*-acting element/myeloid cell specific promoter-heterologous gene constructs can be used to screen for and identify regulators of cell specific transcription. For example, myeloid cells may be transfected with DNA constructs containing functional portions of a myeloid cell specific promoter or *cis*-acting elements and a heterologous gene in the presence of a variety of potential transcription factors; the ability of the transcription factors to alter the function of the promoter or the *cis*-acting element may then be tested by assaying for alterations in expression of the heterologous gene.

The promoter(s) and *cis*-acting element(s) of the present invention may be used in a number of applications. These promoter(s) and *cis*-acting element(s) provide a means of insuring that a selected product, such as a diagnostic, therapeutic or prophylactic substance, is expressed from a myeloid cell type *in vivo*. Therefore, the present invention is useful, for example, for gene therapy or to drive the expression of antiviral agents, such as anti-HIV constructs.

The present invention also provides novel promoters which may used in the production of cancer vaccines.

The present method is also useful in research, for example, to test the effect of the specific expression of heterologous genes, such as oncogenes, in specific myeloid cell types.

Cells of the present invention are also useful for screening compounds for their effect on myeloid cell specific gene expression.

This invention is illustrated further by the following nonlimiting Examples. All of the references listed in the application are intended to be incorporated by reference.

Example 1 - Methodologies and Materials For Identification of CD11d Promoter

The following methodologies and materials were used in the following Examples:

A. Cell Culture

The cell lines used were: THP-1 (acute monocytic leukemia, ATCC TIB-202), HL60 (promyelocytic leukemia, ATCC CCL 240), IM-9 (B-cell multiple myeloma, ATCC CCL-159), Jurkat (T-cell acute leukemia, ATCC TIB 152), MCF-7 (breast adenocarcinoma, ATCC HTB-22) and Schneider's *Drosophila* 2 (D. Melanogaster embryo, ATCC CRL 1963). THP-1, HL60, and Jurkat cells were grown in RPMI-1640 medium containing 10% fetal calf serum (Biofluids, Rockville, MD). IM-9 cells were grown in RPMI-1640 medium containing 20% fetal calf serum (Biofluids, Rockville, MD). HeLa and MCF-7 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Biofluids, Rockville, MD). *Drosophila* Schneider 2 cells were grown in Schneider's medium containing10% insect-tested fetal calf serum (Sigma, St. Louis, MO). All media contained 100 U\ml of penicillin and 100 U\ml streptomycin.

B. Plasmids

Sub B8

A series of 5'-unidirectional deletions of the -946 to +74 region of the CD11d promoter were prepared by the polymerase chain reaction (PCR) with oligonucleotide primers specific to this region and fused to the firefly luciferase

gene (*luc*) in plasmid pGL3-Basic (Promega Corp., Madison, WI). The foreward and reverse primers used in the PCR contained XhoI and HindIII restriction sites, respectively, for cloning of the final PCR product into pGL3-Basic. The -500 to +93 region of the CD11a promoter, the -500 to +50 region of the CD11b promoter, and the -196 to +30 region of the CD11c promoter were prepared in a similar manner and ligated into pGL3-Basic by the methods of Noti *et al.* and Noti. Noti, J. D., *et al.*, Mol. Cell. Biol. 16:2940-2950 (1996); Noti, J. D., J. Biol. Chem. 272:24038-24045 (1997).

A primer containing a deletion of the Sp1-binding site (-63 to -40) was used in the PCR to construct reporter plasmid CD11d(-173/+74)(Δ-63/-40)-*luc*. The plasmid pPacSp1, which expresses Sp1 from the *Drosophila* actin promoter, and the control plasmid pPacO, containing only the *Drosophila* actin promoter, were additionally utilized. The construction of plasmids that express Sp2 and Sp3 from the actin promoter (plasmids pPacSp2 and pPacSp3, respectively) were prepared as described by Noti, J. D., J. Biol. Chem. 272:24038-24045 (1997). The integrity of all constructs was verified by DNA sequence analysis.

C. Transfections and Reporter Assays

Transfections of human cells were performed by electroporation as taught by Noti *et al.*, DNA and Cell Biol., 11:123-138 (1992). The transfected cells were analyzed with the Dual-Luciferase Reporter Assay System (Promega Corp., Madison, WI). Approximately 1 x $10^7$ cells of each leukocyte line or 2 x $10^6$ of MCF-7 cells were co-transfected with 15 μg of each firefly luciferase reporter plasmid and 5 μg of pRL-SV40 Vector (Promega Corp., Madison, WI). The pRL-SV40 vector contains *Renilla* luciferase driven by the early SV40 enhancer/promoter. This vector provided an internal control in which to normalize expression from each firefly luciferase reporter. Electroporated cells were transferred to tissue culture dishes containing 15 ml of medium, and, under certain conditions, phorbol 12-myristate 13-acetate (PMA,10 ng\ml final concentration) was added one hour later. The cells were harvested 24 hr. post-transfection and

luciferase activity was assayed. Firefly luciferase light output was measured in a LB96V-2 Wallac Berthold plate luminometer and normalized against *Renilla* luciferase from the co-transfected vector or against the total protein concentration in the cellular extract. DNA was introduced into *Drosophila* cells by calcium phosphate-mediated transfection as taught by Noti *et al.*, Mol. Cell. Biol., 16:2940-2950 (1996). Approximately 3 x $10^6$ *Drosophila* cells were transfected with 15 $\mu$g of a specific luciferase reporter plasmid and 5 $\mu$g of pPacSp1, pPacSp2, or pPacSp3. The total volume of the plasmid transfection mix was adjusted to 30 $\mu$g with the empty cassette plasmid pPacO. The calcium phosphate-DNA precipitates were left on the cells for 48 hr. prior to harvesting and assaying for luciferase activity. Most transfections were performed in triplicate and repeated two to three times to ensure reproducibility. Statistical analysis was performed using Microsoft Excel (Microsoft Corp, Roselle, IL). Data from individual experiments were pooled and expressed as the mean +/- standard deviation (S.D.).

D. *In Vitro* DNase I Footprinting Analysis

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The PCR was performed to prepare a double-stranded probe to the -173 to +74 region and one primer was labeled with [$\gamma^{32}$P]ATP. The probe was purified by electrophoresis through a 2% agarose gel on to NA45-DEAE paper according to the manufacturer's instructions (Schleicher and Schuell, Keene, NH). Approximately 1-2 x $10^5$ cpm of probe (1-2 ng), and either 50 $\mu$g crude nuclear extract protein (prepared according to Noti *et al.*, DNA and Cell Biol. 11:123-138 (1992)) or 1 to 4 footprinting unit (fpu, concentration determined by the manufacturer) (Promega Corp., Madison, WI) of purified Sp1 protein, were incubated in a total volume of 50 $\mu$l binding buffer containing 0 or 5 $\mu$g poly d(I-C), 6.25 mM $MgCl_2$, 50 mM KCl, 0.5 mM EDTA, 10% glycerol, 0.5 mM DTT, and 25 mM Tris-HCl, pH 8.0, for 15 min. at room temperature. Then 50 $\mu$l of 5 mM $CaCl_2$\10 mM $MgCl_2$, and 0.2-2 units of DNase I were added. After 1 min. at room temperature the reaction was stopped with 90 $\mu$l of 0.2 M NaCl, 0.03 M EDTA,

1% SDS, 10 μg *Escherichia coli* tRNA, phenol\chloroform extracted, ethanol precipitated, and analyzed on a sequencing gel.

*E. In Vivo* Footprinting Analysis

The genomic DNAs from HL60, Jurkat, and IM-9 cells were purified from lysed cells by treatment with proteinase K followed by extensive phenol\chloroform extractions as taught by Mueller *et al., In* Current Protocols in Molecular Biology (F.A. Ausubel *et al.,* eds.) pp. 15.5.1-15.5.26 (Greene Publishing and Wiley Interscience, NY) (1995). The genomic DNAs were treated either *in vivo* or *in vitro* with dimethyl sulfate (DMS), cleaved with piperidine, and analyzed by ligation-mediated PCR as described by Noti *et al.,* Mol. Cell. Biol., 16:2940-2950 (1996) and Mueller *et al., In* Current Protocols in Molecular Biology (F.A. Ausubel *et al.,* eds.) pp. 15.5.1-15.5.26 (Greene Publishing and Wiley Interscience, NY) (1995). The unidirectional linker was composed of two oligonucleotides,

5' GCGGTGATCCCGGGTGATCTGAAT 3' (SEQ ID NO:3) and 5' ATTCAGATCA 3' (SEQ ID NO:4). For footprinting the non-coding strand, the gene-specific primers corresponded to the following regions of the CD11d promoter: primer #1,

5' CTGGGAGAAGGAAGCCAGGTC 3' (SEQ ID NO:5) (for first strand synthesis from the denatured DNAs), which spanned the region -171 to -151; primer #2,

5' CAGGTTGTGGAGGGGGACAGAATGAGG 3' (SEQ ID NO:6) (amplification primer), which spanned the region -146 to -120; and primer #3,

5' GGTTGTGGAGGGGGACAGAATGAGGGTTTTTCC 3' (SEQ ID NO:7) (labeling primer), which spanned the region -144 to -112. First strand synthesis was done for 30 min. at 60°C. The DNAs were denatured for 4 min. at 95°C and amplified by the PCR (18 cycles) as follows: 1 min. at 95°C, 2 min. at 68°C, and 3 min. at 76°C. An extra 5 seconds was added to each extension step and the final extension proceeded for 10 min. Two additional cycles of the PCR were

carried out to label the PCR products as follows: 1 min. at 95°C, 2 min. at 69°C, and 10 min. at 76°C. An approximately equal amount of each sample was loaded on a sequencing gel. The band intensities were analyzed on a Storm Phosphoimager.

F. RNase Protection, S1 Nuclease Analysis, and Primer Extension Analysis

The -381 to +74 region of CD11d was amplified by the PCR and cloned into the XhoI and HindIII sites of pGEM-7Zf(-) (Promega Corp., Madison, WI). This clone was linearized with XhoI, and used as template in an *in vitro* transcription system to prepare a 562 bp RNA probe (Riboprobe, Promega Corp., Madison, WI) that spanned this region according to the manufacturer's instructions. The RNA probe was labeled with [$\alpha$-$^{32}$P]UTP to a specific activity of 7 x $10^7$ cpm/$\mu$g, loaded on to a 5% polacrylamide/8 M urea gel, and subsequently eluted into buffer containing 0.5 M ammonium acetate/1 mM EDTA/0.2% SDS. Approximately 1-2 x $10^5$ cpm of probe was annealed to either 20 $\mu$g total RNA from HL60 or yeast cells and hybridized in 20 $\mu$l of 80% deionized formamide/100 mM sodium citrate pH 6.4/300 mM sodium acetate pH 6.4/1mM EDTA for 16-18 hr. at 44°C. Following hybridization, the annealed probe/RNA complexes were treated with various concentrations of RNase A/T1 (Ambion, Inc., Austin, TX), extracted with proteinase K and phenol/chloroform, and analyzed on a 5% polyacrylamide/8 M urea gel.

For S1 nuclease analysis, a 247 nucleotide single-stranded DNA probe corresponding to the -173 to +74 region of CD11d was prepared by extension of a 22 nucleotide long primer on the luciferase reporter plasmid containing this region. The probe was end-labeled with [$\gamma$-$^{32}$P]ATP (probe specific activity of 5.3 X $10^7$ cpm/$\mu$g). Hybridization of the DNA probe, prepared with the Prime-A-Probe Kit (Ambion, Inc., Austin, TX), to either 500 ng THP1 poly (A+) RNA or 20 $\mu$g yeast total RNA and subsequent digestion with S1 nuclease was performed according to the instructions in the S1-Assay Kit provided by the manufacturer (Ambion Inc, Austin, TX). A second antisense DNA probe, 99

nucleotides long with the 5'-end positioned 19 bp upstream of the ATG codon, was chemically synthesized, end-labeled with [$\gamma$-$^{32}$P]ATP (probe specific activity of 2.88 x $10^8$ cpm/$\mu$g) and hybridized with HL60 poly (A+) RNA or yeast total RNA.

Primer extension analysis was performed essentially as described by Noti *et al.*, DNA and Cell Biol., 11:123-138 (1992). 2.5 ng of a primer labeled with [$\gamma$-$^{32}$P]ATP (specific activity of 1 x $10^9$ cpm/$\mu$g) was hybridized to either 500 ng of HL60 poly (A+) RNA, 25 $\mu$g HL60 total RNA, or 25 $\mu$g yeast total RNA in 15 $\mu$l of 150 mM KCl/10 mM Tris-Cl, pH 8.3 for 3 hr. at 50°C. Then, 30.5 $\mu$l of a solution containing 29 mM Tris-Cl, pH 8.3 at 42°C/14.72 mM $MgCl_2$/8 mM DTT/6.75 mg actinomycin D/0.2 mM each dATP, dTTP, dGTP, dCTP/20 units placental RNase inhibitor/2.5 units MuLV reverse transcriptase was added and incubation continued for 1 hr. at 42°C. The probe/RNA complexes were digested with RNase A/T1 (Ambion, Inc., Austin, TX), extracted with phenol/chloroform, and analyzed on a 5% polyacrylamide/8 M urea gel.

Example 2 – Isolation and Sequence Analysis of The CD11d 5' Flanking Region

The inventors isolated a genomic clone for CD11d that contains the intergenic region between CD11c and CD11d and the 5'-coding portion of CD11d. Utilizing this genomic clone, the regulatory mechanisms for CD11d expression were determined and the clone analyzed in order to locate the CD11d promoter and *cis*-acting elements. CD11d is positioned no more than 11.5 kb downstream of CD11c.

A collection of CD11c positive cosmid clones was re-screened for the presence of CD11d. One clone was completely sequenced and contains exons 15-30 of CD11c, and exons 1-6 of CD11d. Exon 7 was located separately on another clone. The genes were arranged in the same orientation, with the translational stop codon of CD11c positioned 11,461 bp upstream of the translational start codon of CD11d, as shown in FIG 2. The sequence of the 1.2 kb 5'-flanking region and exons 1-7 are shown in FIG. 1.

DNA sequence analysis of this clone showed that the ATG translational codon for CD11d lies approximately 11,460 bp downstream of the

translational stop codon for CD11c and that both genes are transcribed in the same direction, as shown in FIG.2.

As illustrated in FIG. 1, the CD11d genomic clone was found to contain exons 1-7 not previously known in the art, which correspond to nucleotides 1-704 of the CD11d coding sequence. Exon 8 and possibly another exon is predicted to lie between nucleotides 704-982 of CD11d, which would be consistent with an average exon size of 100-150 nucleotides.

Example 3 - Determination of the Transcriptional Start Site of the CD11d Gene

Poly (A+) RNA and total RNA from myeloid HL60 cells was subjected to primer extension analysis with primers N379 (antisense primer with 5'-end positioned 20 nucleotides downstream of the ATG start site) and N470 (antisense primer with 5'-end positioned immediately upstream of the ATG start site), respectively. The longest extension product obtained with N379 was 101 nucleotides long, positioning the transcriptional start site 78 nucleotides upstream of the ATG site. Two extension products, 92, and 93 nucleotides long, were observed with N470 total RNA and would position the transcriptional start site(s) 14 and 15 bp further upstream. No extension products were detected when total yeast RNA was analyzed.

An S1 nuclease protection assay was performed with a 247 nucleotide single-stranded DNA probe that was generated by extension of [$\gamma$-$^{32}$P]ATP-labeled N470 primer on a double-stranded DNA template with exonuclease-free Klenow polymerase. Hybridization of the probe with myeloid THP1 poly (A+) RNA and subsequent S1 nuclease digestion produced four major protected fragments 75-79 nucleotides long and with varying intensities. These results would, therefore, position the start of transcription 75-79 bp upstream of the ATG codon. No protected fragments were produced when the probe was hybridized to yeast total RNA. A second antisense DNA probe, 99 nucleotides long with the 5'-end positioned 19 nucleotides upstream of the ATG codon, was chemically synthesized, end-labeled with [$\gamma$-$^{32}$P]ATP and hybridized with HL60 poly (A+) RNA. S1

nuclease digestion produced four major protected fragments 49-52 nucleotides long, and the most intense fragment was 51 nucleotides long. This second S1 analysis positioned the start of transcription 69 bp upstream of the ATG codon. No protected fragments were produced when the probe was hybridized to yeast total RNA.

An RNase protection assay was performed with a 562 nucleotide long RNA probe prepared by *in vitro* transcription and uniformly-labeled with [$\alpha$-$^{32}$P]UTP. The probe included the 455 nucleotides immediately upstream from the ATG codon. Hybridization of the RNA probe with total RNA from HL60 cells and subsequent digestion with RNase A/T1 produced four protected fragments 71-74 nucleotides long. The length of the two most intense fragments would position the transcriptional start site 72 or 74 bp upstream from the ATG codon. No protected fragments were produced when the probe was hybridized to yeast total RNA.

Taken together, these results position two transcriptional start sites 69-79 bp and 91-92 bp upstream from the ATG codon. RNase protection assays, which provide for the most stringent hybridization and digestion conditions, and which were repeated five times, consistently confined transcription to within 71-74 bp upstream of the ATG codon. No TATA box is present, and transcription is most probably determined by an initiator (Inr) control element which is found in the CD11a and CD11c genes. Since the 69 to 79 bp region shows homology to the classical Inr, and the largest RNase-protected fragment is 74 nucleotides long, we have assigned the thymidine 74 bp upstream from the ATG codon as the major site (+1) of CD11d transcription. FIG. 3 shows the determination of the transcriptional start site for the CD11d gene with a schematic summarizing the sequencing gel information for the results of the transcriptional assays.

Example 4 - Functional Analysis of the CD11d Promoter

CD11d is expressed predominately on myeloid cells and exposure to phorbol ester led to its downregulation from the cell surface. THP1 cells were

transfected with construct CD11d(-946/+74)-*luc*, which contains the -946 to +74 region of CD11d fused to the luciferase gene, and 24 hr. post-transfection were exposed to PMA for varying times, as shown in FIG. 4. Transfected THP1 cells exposed to PMA for up to 10 hr. showed no decrease in luciferase expression, however, after 24 hr., luciferase activity decreased 55%, as shown in FIG. 4. For comparison, expression of CD11a, which is detected in all leukocytes, and CD11c, which is predominately detected in myeloid cells, were monitored following transfection of the CD11a-*luc* and CD11c-*luc* constructs, respectively. Luciferase activity from CD11a-*luc* in THP1 cells was increased 4.5-fold, and luciferase activity from CD11c-*luc* was increased 8.3-fold in the presence of PMA, as illustrated in FIG. 4. These results show that chronic, rather than acute, exposure to PMA leads to downregulation of CD11d expression (and upregulation of CD11a and CD11c expression as expected), and that one or more *cis*-acting elements within the -946 to +74 region mediates this effect.

The -946 to +74 region was further examined to localize the *cis*-acting element(s) responsible for PMA-induced downregulation of CD11d and/or other elements that influence either basal or cell-specific expression. A series of CD11d reporter constructs containing progressively larger 5'-deletions was prepared and transfected into various cell lines. Luciferase expression from the constructs transfected into THP1 cells varied, but not significantly.

CD11d(-173/+74)-*luc*, which contains only the -173 to +74 region of CD11d, retained all of the activity obtained with CD11d(-946/+74)-*luc* and was 43-fold higher than that obtained with the promoterless pGL-3 Basic plasmid, as shown in FIG. 5A. In contrast, luciferase expression in MCF-7 breast cancer cells transfected with these constructs was increased only 2.5 to 6.4-fold over that obtained with pGL-3 Basic as shown in FIG. 5B. Expression from CD11d(-946/+74)-*luc* transfected into the B-cell line IM-9 was also reduced, as luciferase activity was only 8.1-fold over that obtained from pGL3-Basic, as illustrated in FIG. 5C. CD11d(-946/+74)-*luc* expression in the T-cell line Jurkat, was also reduced, although expression was higher than anticipated as it was 18-fold

greater than that obtained from pGL3-Basic, as shown in FIG. 5D. Deletion of the -591 to -378 region resulted in significant increases in luciferase expression in both IM-9 and Jurkat cells to approximately the level of expression of CD11d(-173/+74)-*luc* in THP1 cells . See FIGS 5C and 5D, compare CD11d(-591/+74)-*luc* with CD11d(-378/+74)-*luc*. This result indicates the presence of a cell-specific silencer. Luciferase expression from each of the CD11d-*luc* 5'-deletion constructs transfected into THP1 cells was reduced to approximately the same extent after exposure to PMA, as shown in FIG. 5A. A similar response to PMA was confirmed in another myeloid cell line, HL60, wherein it was discovered that PMA reduces expression in HL60 by 77%. This shows that a PMA-responsive *cis*-acting element(s) lies within the -173 to +74 region, since CD11d(-173/+74)-*luc*, which contains only this region, responds to PMA. In contrast, luciferase expression in IM-9 and Jurkat cells transfected with the CD11d-*luc* 5'-deletion constructs was not reduced by PMA, but instead, was increased 1.5 to 2.5-fold, see FIGS. 5C and 5D. Together, these results show two regions of CD11d that regulate its expression. The -591 to -378 region may contain a cell-specific silencer element, and the -173 to +74 region regulates cell-specific downregulation of CD11d by PMA .

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Example 5 – Sp1 Binding to CD11d Promoter

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DNase I footprint analysis was performed to determine whether DNA binding proteins interact with the -173 to +74 region. When nuclear extracts prepared from unstimulated and PMA-stimulated THP1 cells were added to a probe labeled on the coding strand, strong protection of the -63 to -40 region was revealed. This same region was also protected by nuclear extracts prepared from unstimulated and PMA-stimulated Jurkat and IM-9 cells. When a probe labeled on the non-coding strand was used, strong protection of an overlapping region, -72 to -45, was detected with unstimulated and PMA-stimulated nuclear extracts from all three cell lines. DNA sequence analysis of the overlapping region revealed the presence of an Sp1 binding site.

*In vitro* DNase I footprint analysis showed that purified Sp1 protein could also protect the -63 to -40 region. Electrophoretic mobility shift analysis (EMSA) with THP1 nuclear extract protein and a probe to the -63 to -40 region revealed a protein/DNA complex that could be supershifted with anti-Sp1 antibody.

Example 6 – Sp1 Binding Region Required For CD11d Promoter Activity

To determine whether the Sp1-binding site is important for CD11d expression, this site was deleted from CD11d(-173/+74)-*luc* and its effect on expression was monitored in transfected cells. Deletion of the -63 to -40 region resulted in reduction of luciferase expression from CD11d(-173/+74)(Δ-63/-40)-*luc* to 24% in transfected THP1 cells, as shown in FIG. 6A. When transfected THP1 cells were exposed to PMA, expression from CD11d(-173/+74)-*luc* was reduced to 30%. Deletion of the -63 to -40 region further reduced luciferase expression from CD11d(-173/+74)(Δ-63/-40)-*luc* in PMA-stimulated THP1 cells only an additional 7%, as shown in FIG. 6A. Luciferase expression from CD11d(-173/+74) (Δ -63/-40)-*luc* in transfected IM-9 and Jurkat cells was similarly reduced to 20% and 24%, respectively, as illustrated by FIGS 6B and 6C. Although PMA did not reduce the expression of luciferase from CD11d(-173/+74)-*luc* transfected into IM-9 and Jurkat cells, expression from CD11d(-173/+74)(Δ-63/-40)-*luc* was reduced to 18% and 20%, respectively, as shown in FIGS. 6B and 6C. These results show that the -63 to -40 region is essential for CD11d promoter activity in both myeloid and non-myeloid cells. Further, the inability of PMA to reduce luciferase expression from CD11d(-173/+74)-*luc* in non-myeloid cells is dependent on the integrity of this region.

Example 7 – Sp1 Regulation of CD11d

To show that CD11d promoter activity is mediated through an interaction of Sp1 with the -63 to -40 region, *Drosophila* cells, which are deficient in Sp-related proteins, were cotransfected with pPacSp1 along with CD11d(-173/+74)-*luc,* as shown in FIG. 7.

The role of two other members of the Sp-family, Sp2 and Sp3, expressed from pPacSp2 and pPacSp3, respectively, was similarly analyzed. Sp1-dependent luciferase activity from the CD11d promoter was shown to increase 4.1-fold in *Drosophila* cells cotransfected with pPacSp1 and CD11d(-591/+74)-*luc,* as shown in FIG. 7A. In contrast, no induction of luciferase activity was seen when either pPacSp2 or pPacSp3 was cotransfected. Analysis of CD11d(-378/+75)-*luc* in cotransfection experiments yielded similar results.

For comparison, the response of the CD11c promoter to pPacSp1 and pPacSp3 is illustrated in FIG. 7B. As known in the art, CD11c promoter activity is dependent on both Sp1 and Sp3, and was indicated by the 19.5-fold and 18.9-fold induction by Sp1 and Sp3, respectively. It is also known in the art that Sp1 and Sp3 compete for the same sites on the CD11c promoter, however, luciferase activity from CD11d(-591/+74)-*luc* is maximal in the presence of pPacSp1 alone, and was not further increased when both pPacSp1 and pPacSp3 were present, as shown in FIG. 7A. Since pPacSp1-dependent expression of luciferase from CD11d(-591/+74)-*luc* is also not decreased in the presence of pPacSp3, Sp3 does not compete with Sp1 for binding to the same site. This suggests that Sp3 does not function as a repressor of CD11d promoter activity, in contrast to its reported repressor-like activity on other promoters.

Example 8 - Myeloid-Specific Downregulation of CD11d by PMA

The above results show that cell-specific downregulation of CD11d promoter activity is mediated through one or more *cis* elements within the -173 to +74 region. The inability of IM-9 and Jurkat cells to maintain CD11d expression in the presence of PMA when the Sp1-binding site was deleted indicated that Sp1 was a necessary factor involved in this response. Further, the possibility that loss of Sp1-binding was linked to downregulation of CD11d promoter activity in THP1 cells exposed to PMA was suggested when the reduction in luciferase activity from CD11d(-173/+74)-*luc* in transfected THP1 cells exposed to PMA was found to be about the same as that obtained in unstimulated THP1 cells transfected with the

Sp1-deleted construct CD11d(-173/+74)(Δ-63/-40)-*luc* (70% vs. 76% reduction, respectively, as shown in FIG. 6.

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*In vivo* genomic footprinting was performed to determine if selective Sp1-binding occurs on the CD11d promoter. Genomic DNA, methylated *in vivo* with dimethyl sulfate, was isolated from HL60, IM-9, and Jurkat cells that were either unstimulated or PMA-stimulated. DNA was also isolated from HL60 and Jurkat cells, stripped of bound protein, and methylated *in vitro* as controls. Analysis of the CD11d non-coding strand, as shown in FIG. 8, revealed hyposensitive sites in unstimulated HL60 DNA at positions -38, -40, -42, -43, -50, -52, -55, -56, -58 to -61, -63, -65, and -66 which correspond to guanine nucleotides in the Sp1-binding site. No protection was seen over these positions on genomic DNA from PMA-stimulated HL60 cells. In contrast, genomic DNAs from IM-9 and Jurkat cells, either unstimulated or PMA-stimulated, were similarly protected over these positions. From these results, we conclude that occupation of the CD11d promoter by Sp1 is significantly reduced in PMA-stimulated myeloid cells, and that myeloid-specific downregulation of CD11d expression is mediated through preferential loss of Sp1 binding following PMA stimulation.

It is understood that the invention is not confined to the particular embodiments set forth herein as illustrative, but embraces all such modified forms thereof as come within the scope of the following claims.